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(54) NOVEL PROTEIN AND DNA THEREOF

(57) The present invention relates to a phosphatonin protein or a salt thereof and the like.

The protein of this invention, a partial peptide thereof or a salt thereof, and DNAs encoding them can be used for obtainment of antibody and antiserum, construction of an expression system of the protein of this invention, screening of a candidate compound for pharmaceutical product using this expression system and the like.

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of phosphatonin reported by Rowe P.S.N. (WO 99/60017) lacked 5' region including the initial methionine, and that its recombinant product showed an function opposite to the physiological function of phosphatonin, a full-length gene sequence of phosphatonin and a gene product thereof have not been clarified yet.

Disclosure of the Invention

[0009] The present invention aims at providing a novel protein having a phosphaturic activity and/or a hypophosphatemia-inducing activity, its partial peptide or its salt, a DNA encoding said protein, recombinant vectors, transformants, methods for manufacturing said protein, pharmaceutical agents comprising said protein or DNA, antibodies against said protein, methods for screening receptor agonist/antagonist and a screening kit of receptor agonist/antagonist, a receptor agonist/antagonist obtained by said screening, methods for screening a compound having an inhibitory action on a proteinase that degrades the protein or a salt thereof, and a screening kit thereof, and a compound obtained by said screening or a salt thereof.

[0010] Isolation of a novel protein having a phosphaturic activity and/or a hypophosphatemia-inducing activity clarifies the onset mechanisms of OHO and XLH and reabsorption mechanism of phosphorus in the kidney, and leads to the development of a new pharmaceutical product useful for the prophylaxis or treatment of various diseases caused by abnormal control of phosphorus concentration in blood.

[0011] As a result of intensive studies done by the present inventors, they have succeeded in cloning cDNA having a novel base sequence, from a cDNA library derived from OHO patients. The present inventors have found that a protein encoded by the obtained cDNA is a novel protein having a phosphaturic activity and/or a hypophosphatemia-inducing activity, and they have studied further based on such finding, which resulted in the completion of the present invention.

[0012] Accordingly, the present invention provides

(1) a protein comprising an amino acid sequence identical or substantially identical to an amino acid sequence consisting of amino acid Nos. 17 - 525 of the amino acid sequence presented by SEQ ID:1, or a salt thereof,
(2) a protein comprising an amino acid sequence consisting of amino acid Nos. 17 - 525 of the amino acid sequence presented by SEQ ID:1, or a salt thereof,
(3) the protein of (1) or a salt thereof, which is a protein comprising an amino acid sequence identical or substantially identical to the amino acid sequence presented by SEQ ID:1, or a salt thereof,
(4) the protein of (3) or a salt thereof, which is a protein having the amino acid sequence presented by SEQ ID:1 or a salt thereof,
(5) the protein of (1)-(4) or a salt thereof, which is a protein having a phosphaturic activity and/or a hypophosphatemia-inducing activity,
(6) the protein of (1)-(4), which is a protein having at least one activity selected from (i) an activity that suppresses a sodium-dependent phosphorous (Na^+-Pi) transport activity in kidney, (ii) an activity that suppresses a 25-hydroxy vitamin D_3-1_α-hydroxylase activity in kidney, and (iii) an activity that promotes a 25-hydroxy vitamin D_3-24-hydroxylase activity in kidney,
(7) a partial peptide of the protein of (1), or a salt thereof,
(8) a DNA comprising a DNA having a base sequence encoding the protein of (1),
(9) the DNA of (8), which has a base sequence presented by SEQ ID:2 or SEQ ID:3,
(10) a recombinant vector comprising the DNA of (8),
(11) a transformant retaining the recombinant vector of (10),
(12) a method for manufacturing the protein of (1), the partial peptide of (7) or a salt thereof, which comprises culturing the transformant of (11) to produce and accumulate the protein of (1) or the partial peptide of (7) and harvesting the same.
(13) a pharmaceutical agent comprising the protein of (1), the partial peptide of (7) or a salt thereof,
(14) a pharmaceutical agent comprising the DNA of (8),
(15) the pharmaceutical agent of (13) or (14), which is capable of regulating and improving abnormal concentration of phosphorus in blood,
(16) an antibody against the protein of (1), the partial peptide of (7) or a salt thereof,
(17) a method for quantifying the protein of (1), the partial peptide of (7) or a salt thereof, which comprises using the antibody of (16),
(18) a method for diagnosing a disease involved by the protein of (1), the partial peptide of (7) or a salt thereof, which comprises using the quantification method of (17),
(19) a method for screening a receptor agonist or antagonist, which comprises using the protein of (1), the partial peptide of (7) or a salt thereof,
(20) a screening kit of a receptor agonist or antagonist, which comprises the protein of (1), the partial peptide of

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(32) or by the use of the screening kit of (23),
(34) a method for screening a compound or a salt thereof that promotes or inhibits intracellular signal transduction after binding of the protein of (1), the partial peptide of (7) or a salt thereof to a receptor, which comprises using the protein of (1), the partial peptide of (7) or a salt thereof,
(35) a screening method of (34), which comprises measuring and comparing intracellular signal transduction after binding of the protein of (1), the partial peptide of (7) or a salt thereof to a receptor, between (i) a case where the protein of (1), the partial peptide of (7) or a salt thereof is brought into contact with a cell containing the receptor, and (ii) a case where the protein of (1), the partial peptide of (7) or a salt thereof and a test compound are brought into contact with the cell containing the receptor,
(36) a screening kit for a compound or a salt thereof that promotes or inhibits intracellular signal transduction after binding of the protein of (1), the partial peptide of (7) or a salt thereof to a receptor, which comprises the protein of (1), the partial peptide of (7) or a salt thereof,
(37) a compound or a salt thereof that promotes or inhibits intracellular signal transduction after binding of the protein of (1), the partial peptide of (7) or a salt thereof to a receptor, which is obtained by the screening method of (34) or (35), or by the use of the screening kit of (36),
(38) a pharmaceutical agent comprising a compound or a salt thereof that promotes or inhibits intracellular signal transduction after binding of the protein of (1), the partial peptide of (7) or a salt thereof to a receptor, which is obtained by the screening method of (34) or (35), or by the use of the screening kit of (36),
(39) the pharmaceutical agent of (38), which is an agent for the prophylaxis or treatment of oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure, hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure or the like, and the like.

Brief Description of the Drawings

[0014] Fig. 1 shows a base sequence of a DNA encoding the phosphatonin protein of the present invention, which is contained in plasmid pCR-PHOS obtained in Example 1 and an amino acid sequence of the phosphatonin protein of the present invention deduced therefrom (continued on Fig. 2).
[0015] Fig. 2 shows a base sequence of a DNA encoding the phosphatonin protein of the present invention, which is contained in plasmid pCR-PHOS obtained in Example 1 and an amino acid sequence of the phosphatonin protein of the present invention deduced therefrom (continued from Fig. 1 onto Fig. 3).
[0016] Fig. 3 shows a base sequence of a DNA encoding the phosphatonin protein of the present invention, which is contained in plasmid pCR-PHOS obtained in Example 1 and an amino acid sequence of the phosphatonin protein of the present invention deduced therefrom (continued from Fig. 2 onto Fig. 4).
[0017] Fig. 4 shows a base sequence of a DNA encoding the phosphatonin protein of the present invention, which is contained in plasmid pCR-PHOS obtained in Example 1 and an amino acid sequence of the phosphatonin protein of the present invention deduced therefrom (continued from Fig. 3).
[0018] Fig. 5 shows hydrophilicity · hydrophobicity of the phosphatonin protein of the present invention obtained in Example 1 as deduced from the amino acid sequence of the protein according to a Kyte Doolittle method.
[0019] Fig. 6 shows a plasmid construct of plasmid pTCII-mPHOS-2 obtained in Example 2.
[0020] Fig. 7 shows an SDS polyacrylamide gel electrophoresis of the phosphatonin protein of the present invention obtained in Example 3.
[0021] Fig. 8 shows HPLC analysis results of the phosphatonin protein of the present invention obtained in Example 3.
[0022] Fig. 9 shows an N-terminal sequence analysis of the phosphatonin protein of the present invention obtained in Example 3.
[0023] Fig. 10 shows an SDS polyacrylamide gel electrophoresis of phosphorylated Phosphatonin obtained in Example 3.
[0024] Fig. 11 shows a calibration curve in Phosphatonin ELISA.
[0025] Fig. 12 shows the amount of the phosphatonin protein of the present invention in human serum.
[0026] Fig. 13 shows a plasmid construct of plasmid pT-PHOSF-11 obtained in Example 6.
[0027] Fig. 14 shows an SDS polyacrylamide gel electrophoresis of the phosphatonin protein of the present invention obtained in Example 7.
[0028] Fig. 15 shows the results of activity determination of phosphatonin protein of Example 8.
[0029] The protein of this invention is a protein having an amino acid sequence identical or substantially identical to the amino acid sequence presented by SEQ ID:1, more preferably a protein having an amino acid sequence identical

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the proteins of this invention including the protein containing the amino acid sequence presented by SEQ ID:1, the C-terminal may be any of carboxyl group (-COOH), carboxylate ($-COO^-$), amide ($-CONH_2$) and ester (-COOR).

[0040] For R in the esters, C_{1-6} alkyl groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl and the like, C_{3-8} cycloalkyl groups such as cyclopentyl, cyclohexyl and the like, C_{6-12} aryl groups such as phenyl, α-naphthyl and the like, C_{7-14} aralkyl groups including phenyl-C_{1-2} alkyl groups such as benzyl, phenethyl and the like, and α-naphthyl-C_{1-2} alkyl groups such as α-naphthylmethyl are used, and pivaloyloxymethyl groups and the like, which are commonly used for oral esters, is also used.

[0041] When the protein of this invention has a carboxyl group (or carboxylate) at a site other than the C-terminal, the proteins having an amidized or esterified caroboxyl group are included in the proteins of this invention. For the ester form in this case, for example, the C-terminal esters described above and the like are used.

[0042] Furthermore, the proteins of this invention also include proteins described above in which the amino group of the N-terminal methionine residue is protected by a protecting group (e.g. C_{1-6} acyl group such as formyl group, acetyl group and the like), those in which the N-terminal is cleaved in vivo and thus produced N-terminal glutamyl group is converted to pyroglutamate, those in which substituents on amino acid side chains in the molecule (e.g. -OH, -SH, amino group, imidazole group, indole group, guanidino group and the like) are protected by appropriate protecting groups (e.g. C_{1-6} acyl group such as formyl group, acetyl group and the like), or complex proteins to which sugar chains are bound, so-called glycoproteins.

[0043] More specifically, as the protein of this invention, for example, a protein derived from tumor of human OHO patients, having a 17th - 525th amino acid sequence of the amino acid sequence presented by SEQ ID:1, a protein derived from tumor of human OHO patients having the amino acid sequence presented by SEQ ID:1, and the like are preferred.

[0044] The 17th - 525th amino acid sequence of the amino acid sequence presented by SEQ ID:1 is a sequence obtained by deleting secretion signal sequence from the amino acid sequence presented by SEQ ID:1.

[0045] The partial peptide of the protein of this invention may be any of peptide having the same activity as that of the aforementioned protein of this invention, for example, phosphaturic activity, hypophosphatemia-inducing activity, Na^+-Pi transport inhibitory activity, 25-hydroxy vitamin D_3-1α-hydroxylase inhibitory activity, 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity in kidney cell and the like.

[0046] Specifically, a partial peptide having the 17th - 525th amino acid sequence, a partial peptide having the 17th - 330th amino acid sequence, a partial peptide having the 331st - 525th amino acid sequence and the like of the amino acid sequence presented by SEQ ID:1 are preferably used.

[0047] As the partial peptide of this invention, moreover, a partial peptide having an amino acid sequence substantially identical to the amino acid sequence presented by SEQ ID:1, and having a substantially same activity as that of a peptide having the amino acid sequence presented by SEQ ID:1 is preferable.

[0048] As the substantially same activity, for example, phosphaturic activity, hypophosphatemia-inducing activity, Na^+-Pi transport activity, 25-hydroxy vitamin D_3-1α-hydroxylase inhibitory activity, 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity in kidney cell and the like are mentioned. By being substantially the same means that the activities are same in terms of properties. Therefore, it is preferable that the phosphaturic activity, hypophosphatemia-inducing activity, Na^+-Pi transport inhibitory activity, 25-hydroxy vitamin D_3-1α-hydroxylase inhibitory activity, 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity in kidney cell be equivalent (e.g., about 0.5 to 2-fold). The levels of these activities and quantitative factors such as molecular weight of protein and the like may be different.

[0049] These partial peptides of this invention also include one that is an (competitive) inhibitory type to the protein of this invention, that is one having an inhibitory activity on the activity of the protein of this invention.

[0050] Moreover, as the partial peptide of the present invention, a partial peptide of a protein having a homology of not less than about 40%, preferably not less than 60%, more preferably not less than about 80%, further preferably not less than about 90%, most preferably not less than about 95%, with the amino acid sequence presented by SEQ ID:1 is used. More specifically, the partial peptide of the protein of this invention includes the partial peptide of the protein containing an amino acid sequence wherein 1 or more (e.g., 1-80, preferably about 1-20, more preferably about 1-9, more preferably several (1 or 2)) amino acids are deleted from the amino acid sequence presented by SEQ ID:1, an amino acid sequence wherein 1 or more (e.g., 1-80, preferably about 1-20, more preferably about 1-9, more preferably several (1 or 2)) amino acids are added to the amino acid sequence presented by SEQ ID:1, an amino acid sequence wherein 1 or more (e.g., 1-80, preferably about 1-20, more preferably about 1-9, more preferably several (1 or 2)) amino acids in the amino acid sequence presented by SEQ ID:1 are substituted by other amino acids, and the like.

[0051] Furthermore, a partial peptide having a homology of not less than about 40%, preferably not less than 60%, more preferably not less than about 80%, more preferably not less than about 90%, most preferably not less than about 95%, with the 17th - 525th amino acid sequence, the 17th - 330th amino acid sequence or the 331st - 525th amino acid sequence (hereinafter these are to be briefly referred to as amino acid sequence A) of the amino acid sequence presented by SEQ ID:1 is used. More specifically, a partial peptide includes an amino acid sequence wherein 1 or more (e.g., 1-80, preferably about 1-20, more preferably about 1-9, more preferably several (1 or 2)) amino acids are

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drazide, tritylhydrazide and the like is used.

[0060] The hydroxyl group of serine can be protected, for example, by esterification or etherification. Groups appropriate for this esterification include, for example, lower alkanoyl groups such as acetyl group, aroyl groups such as benzoyl group, and groups derived from carbon such as benzyloxycarbonyl group and ethoxycarbonyl group. Groups appropriate for etherification include, for example, benzyl group, tetrahydropyranyl group, t-butyl group and the like.

[0061] As a protecting group of the phenolic hydroxyl group of tyrosine, for example, BzL, Cl_2-Bzl, 2-nitrobenzyl, Br-Z, and tertiary butyl or the like are used.

[0062] As a protecting group of the imidazole of histidine, for example, Tos, 4-methoxy-2,3,6-trimethylbenzen sulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, Fmoc or the like are used.

[0063] For activated carboxyl groups in the starting material, for example, corresponding acid anhydride, azide, active ester [ester formed with alcohol (e.g. pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, para-nitrophenol, HONB, N-hydroxysuccimide, N-hydroxyphthalimide, HOBt)], or the like are used. Activated amino groups used in the starting material include, for example, corresponding phosphoric amide.

[0064] For the method for removing (eliminating) the protecting groups, catalytic reduction in hydrogen gas flow in the presence of a catalyst such as Pd-black, Pd-carbon and the like, acid treatment with hydrogen fluoride anhydride, methanesulfonic acid, trifluoromethanesulfonic acid, and trofluoroacetic acid, and mixture of these acids, basic treatment with diisopropylethylamine, triethylamine, piperidine, piperazine, and the like, reduction by sodium in liquid ammonia, and the like are used. The elimination reaction by the acid treatment described above is generally performed at a temperature ranging from about -20°C to 40°C. In acid treatment, addition of a cation scavenger such as anisole, phenol, thioanisole, metacresol, paracresol, dimethylsulfide, 1,4-butanedithiol, and 1,2-ethanedithiol is effective. 2,4-dinitrophenyl group used as the protecting group of the imidazole of histidine is removed by treatment with thiophenol. Formyl group used as the protecting group of the indole of tryptophan is removed by the acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol and the like, as well as alkaline treatment with diluted sodium hydroxide solution, diluted ammonia and the like.

[0065] Protection of functional groups that should not be involved in the reaction of the starting materials, protecting groups, elimination of the protecting groups, activation of functional groups involved in the reaction, and the like may be appropriately selected from publicly known groups and means.

[0066] In another method for obtaining amide form of the proteins, for example, first, the α-carboxyl group of the carboxy termial amino acid is protected by amidation, and the peptide (protein) chain is extended for a desired chain length from the amino group side. Then, a protein in which only the protecting group of the N-terminal α-amino group was removed from said peptide and a protein in which only the protecting group of the C-terminal carboxyl group is removed are produced. These two proteins are condensed in the mixed solvent described above. The details of the condensation reaction are the same as described above. After the protected protein obtained by condensation is purified, all protecting groups are removed by the method described above, and the desired crude protein is obtained. The desired protein in amide form can be obtained by purifying this crude protein using various known means and by lyophilizing the major fraction.

[0067] To obtain esterified form of the protein, the α-carboxyl group of the carboxy termial amino acid is condensed with a desired alcohols to prepare amino acid ester, and the desired esterified form of the protein can be obtained by the same procedure as in the preparation of the amide form of the protein.

[0068] The partial peptides or its salts of this invention can be manufactured by publicly known method for peptide synthesis or by cleaving the protein of this invesntion with appropriate peptidase. For the method for peptide synthesis, for example, either solid phase synthesis or liquid phase synthesis may be used. The partial peptides or amino acids that may compose the protein of this invention are condensed with the residual portion. When the product has protecting groups, the desired peptide can be obtained by eliminating the protecting groups. The publicly known condensation and elimination of protecting group include the methods described in 1) - 5) below.

1) M. Bodanszky and M.A. Ondetti: Peptide Synthesis. Interscience Publishers, New York (1966)
2) Schroeder and Luebke: The Peptide. Academic Press, New York (1965)
3) N. Izumiya, et al.: Basics and experiments of peptide synthesis, Maruzen Co. (1975)
4) H. Yajima and S. Sakakibara: Biochemical Experiment 1, Chemistry of Proteins IV, 205 (1977)
5) H. Yajima ed.: A sequel to Development of Pharmaceuticals Vol. 14, Peptide Synthesis, Hirokawa Shoten

[0069] After the reaction, moreover, the protein of this invention are purified by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography, recrystallization and the like. When the protein obtained by the above methods is free form, it can be converted to an appropriate salt form by known methods. On the oher hand, when a salt form is obtained, it can be converted to the free form by known methods.

[0070] For the DNA encoding the protein of this invention, any DNA containing the base sequence encoding the

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[0084] In addition to the vectors described above, expression vectors contaninig enhancer, splicing signal, polyA additive signal, selection marker, and SV40 replication origin (hereinafter sometimes to be briefly referred to as SV40 ori) may be used when desired. For the selection marker, for example, dihydrofolate reductase (hereinafter sometimes to be briefly referred to as dhfr) gene [methotrexate (MTX)-resistant], ampicillin resistance gene (hereinafter sometimes to be briefly referred to as Amp^r), neomycin resistance gene (hereinafter sometimes to be briefly referred to as Neo, G418-resistant) and the like are used. Especially, when dhfr gene is used as the selection marker using CHO (dhfr$^-$) cells, the objective gene can be selected using thymidine-free medium.

[0085] When necessary, a signal sequence appropriate for the host is added to the N-terminal of the protein of this invention. When the host is bacteria of *Escherichia genus,* PhoA signal sequence, OmpA signal sequence and the like are used. When the host is bacteria of *Bacillus genus,* α-amylase signal sequence, subtilisin signal sequence and the like are used. When the host is yaest, MF_α signal sequence, SUC2 signal sequence and the like are used. When the host is animal cells, for example, insulin signal sequence, $_\alpha$-inetrferon signal sequence, the signal sequence of antibody molecule and the like can be respectively used.

[0086] Introducing the vectors containing the DNA encoding the protein of this invention constructed as described above to cell, transformants can be manufactured.

[0087] For the host, for exmaple, *Escherichia genus, Bacillus genus,* yeast, insect cells, insects, animal cells and the like are used.

[0088] Specific examples of the host of *Escherichia genus* are *Escherichia coli* K12 DH1 [Proceedings of the National Academy of Sciences of the USA (Proc. Natl. Acad. Sci. USA) Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology Vol. 120, 517 (1978)], HB101 [Journal of Molecular Biology Vol. 41, 459 (1969)], C600 [Genetics Vol. 39, 440 (1954)] and the like are used.

[0089] For the host of Bacillus genus, for example, *Bacillus subtilis* MI114 [Gene Vol. 24, 255 (1983)] and 207-21 [Journal of Biochemistry Vol. 95, 87 (1984)] and the like are used.

[0090] For the host of yeast, for example, *Saccharomyces* cerevisiae AH22, AH22R$^-$, NA87-11A, DKD-5D, 20B-12, *Schizosaccharomyces* pombe NCYC1913, NCYC2036, *pichia pastoris* and the like are used.

[0091] For the host of insect cells, for example, when the virus is AcNPV, Spodoptera frugiperda cells (Sf cells), MG1 cells derived from the middle gut of Trichoplusia ni, High Five™ cells derived from Trichoplusia ni eggs, Mamestra brassicae-derived cells, Estigmena acrea-derived cells or the like are used. When the virus is BmNPV, silkworm-derived cells Bombyx mori N (BmN cells) are used. For said Sf cells, for example, SF9 cells (ATCC CRL1711), Sf21 cells (Vaughn, J.L. et al., In Vitro 13, 213-217 (1977)) or the like are used.

[0092] For the host of insect, for example, silkworm larvae or the like are used [Maeda et al., Nature, Vol. 315, 592 (1985)].

[0093] For animal cells, for example, monkey COS-1, COS-7, Vero cell, Chinese hamster cells CHO (hereinafter to be briefly referred to as CHO cells), dhfr gene-deficient Chinese hamster cell CHO (hereinafter to be briefly referred to as CHO (dhfr$^-$) cells), L cells, myeloma cells, human FL cells, 293 cells, C127 cells, BALB3T3 cells, Sp-2/O cells or the like are used. Among them, CHO cells, CHO(dhfr$^-$) cells, 293 cells and the like are preffered.

[0094] For transformation of bacteria of *Escherichia genus,* for example, the methods published in Proc. Natl. Acad. Sci. USA Vol. 69, 2110 (1972), Gene Vol. 17, 107 (1982), and the like are used.

[0095] Bacteria of *Bacillus genus* can be transformed according to, for example, the method published in Molecular & General Genetics Vol. 168, 111 (1979) and the like.

[0096] Yeast can be transformed according to, for example, the methods published in Methods in Enzymology Vol. 194, 182-187 (1991).

[0097] Insect cells and insects can be transformed according to, for example, the method published in Bio/Technology, 6, 47-55 (1988) and the like.

[0098] Animal cells can be transformed by, for example, the methods described in Cell Technology (Saibo Kogaku) Separate Vol. 8, New Cell Technology Experimental Protocol, 263-267 (1995) (Shujun-sha).

[0099] As a method for introducing an expression vector into a cell, for example, calcium phosphate method [Graham F. L. and van der Eb A. J., Virology, 52, 456-467 (1973)], DEAE-dextran method [Sompayrac L.M. and Danna K.J., Proc. Natl. Acad. Sci. USA, 78, 7575-7578, 1981], lipofection method [Malone R.W. et al., (Proc. Natl. Acad. Sci. USA, 86, 6077-6081, 1989)], electroporation method [Nuemann E. et al., EMBO J., 1, 841-845 (1982)] and the like can be mentioned.

[0100] In this way, a transformant transformed with an expression vector containing a DNA encoding the protein of this invention can be obtained.

[0101] As a method for stably expressing the protein of this invention using animal cells, a method comprising selection of a cell by clonal selection, wherein an expression vector introduced into the above-mentioned animal cell is incorporated into chromosome, can be mentioned. Specifically, a transformant can be selected using the above-mentioned selection marker as an index,. Moreover, repeated clonal selections of animal cells obtained by using a selection marker give a stable animal cell line having highly capable of expressing the protein of this invention.

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the salt can be converted to the free form or other salt by publicly known methods or its modified methods.

[0116] The protein produced by transformants can be optionally modified or the partial polypeptide can be removed from the protein by treating the protein with an appropriate protein-modifying enzyme before or after purification. For the protein-modifying enzyme, for example, trypsin, chymotrypsin, arginylendopeptidase, protein kinase, glycosidase and the like are used.

[0117] The existence of the protein of this invention produced as described above can be detected by an enzyme immunoassay using specific antibody, and the like.

[0118] Antibodies against the protein, its partial peptides, and their salts of this invention may be either polycloncal antibodies or a monoclonal antibody that recognize the protein of this invention, its partial peptides, and their salts (hereinafter sometimes to be briefly referred to as the protein of this invention).

[0119] Antibodies against the protein of this invention (hereinafter sometimes to be briefly referred to as the antibody of this invention) can be manufactured according to publicly known methods for manufacturing antibodies and antiserum using the proteins of this invention as antigens.

[Production of a monoclonal antibody]

(a) Establishment of monoclonal antibody-producing cells

[0120] The protein of this invention is administered with or without carrier and diluent to sites by which antibody production is induced in warm-blooded animals. To increase the productivity of antibodies, Freund's complete or incomplete adjuvant may be administered. Usually, the administration is performed every 2 - 6 weeks, 2 - 10 times in total. Warm-blooded animals used include monkeys, rabbits, dogs, guinea pigs, mice, rats, sheeps, goats and chickens. Among them, mice and rats are preferably used.

[0121] In production of monoclonal antibody-producing cells, animals in which antibody titer are observed are selected from warm-blooded animals immunized with antigen such as mice, and the spleen or lymph nodes are excised 2 - 5 days after the final immunization. Monoclonal antibody-producing hybridomas can be produced by fusing the antibody-producing cells contained in the excised organ with myeloma cells. Antibody titer in an antiserum can be measured by, for example, reacting the labeled protein described below with the antiserum, followed by measurement of the activity of the label bound to the antibodies. The cell fusion can be performed according to known methods such as the method by Köhler and Milstein [Nature Vol. 256, 495 (1975)]. As the fusion-promoting agent, for example, polyethyleneglycol (PEG) and Sendai virus are used, and PEG is preferred.

[0122] For myeloma cells, for example, NS-1, P3U1, SP2/0, AP-1 and the like are included, and P3U1 is preferably used. The preferable ratio of the number of antibody-producing cells (spleen cells) to that of myeloma cells is about 1: 1 - 20:1. PEG (preferably PEG1000 - PEG6000) is added at the concentration of about 10 - 80% and the cells are incubated at about 20 - 40°C, preferably about 30 - 37°C, for about 1 - 10 minutes, then, an efficient cell fusion can be performed.

[0123] Various methods can be used for screening monoclonal antibody-producing hybridomas. For example, hybridoma culture supernatant is added to the proteinous antigen adsorbed on a solid phase (e.g. microplate) directly or with carrier, and anti-immunoglobulin antibody labeled with radioactive substance, enzyme or the like (when the cells for fusion are mouse cells, anti-mouse immunoglobin antibody is used) or protein A is added, then the monoclonal antibody bound to the solid phase is detected. In other method, hybridoma culture supernatant is added to anti-immunoglobulin antibody or protein A adsorbed on a solid phase, and the protein labeled with radioactive substance, enzyme or the like is added, then the monoclonal antibody bound to the solid phase is detected.

[0124] Monoclonal antibody can be selected by publicly known methods or its modified methods. Usually, medium for animal cells supplemented with HAT (hypoxanthine, aminopterin, thymidine) can be used. Any medium in which hybridoma can grow is used for selection and clonal growth. For example, RPMI 1640 medium containing 1 - 20%, preferably 10 - 20% fetal calf serum, GIT medium (Wako Pure Cheimcal Industries, Ltd.) containing 1 - 10% fetal calf serum, serum-free medium for hybridoma culture (SFM-101, Nissui Pharmaceutical Co., Ltd.) and the like can be used. The temperature for culture is usually 20 - 40°C, preferably about 37°C. The duration of culture is usually 5 days to 3 weeks, preferably 1 - 2 weeks. Usually, the cells can be cultured under 5% CO_2 gas. The antibody titer in hybridoma culture supernatant can be measured by the same procedure as that for antibody titer in antiserum described above.

(b) Purification of monoclonal antibody

[0125] The monoclonal antibodies can be separated and purified by the publicy known methods, for example, the methods for purification of the immunoglobulins [e.g. salting out, alcohol precipitation, isoelectric precipitation, electrophoresis, adsorption-desorption method using ion exchangers (e.g. DEAE), ultracentrifugation, gel filtration, specific purification methods in which only antibody is collected using an active adsorbent such as antigen-bound solid phase,

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affords a suitable dose within the indicated range. When the DNA of this invention is used, it can be administered according to a conventional method as the DNA alone or after insertion into a suitable vector such as retrovirus vector, adenovirus vector, adenovirus associated viral vector and the like.

[0138] For the additive that can be mixed in tablets, capsules and the like, for example, binders such as gelatin, cornstarch, tragacanth, and gum arabic, excipients such as crystalline cellulose, imbibers such as cornstarch, gelatin, alginic acid and the like, lubricants such as magnesium stearate, sweeteners such as sucrose, lactose and saccharin, and flavors such as peppermint, akamono oil and cherry are used. When the unit-dosage form is capsule, liquid carrier such as oils and fat can be contained. Aseptic compositions for injection can be formulated according to the usual preparation procedure such as dissolving or suspending the active substance in vehicle, e.g. water for injection, natural plant oils e.g. sesame oil and coconut oil and the like. For the aqueous solution for injection, for example, physiological saline and isotonic solutions containing glucose and other supplement (e.g. D-sorbitol, D-mannitol, sodium hydrochloride and the like)are used. Appropriate dissolution-assisting agents, for example, alcohol (e.g. ethanol and the like), polyalcohol (e.g. propylene glycol, polyethylene glycol and the like), nonionic surfactant (e.g. polysorbate 80™, HCO-50 and the like) and the like may be used in combination. For the oily solution, for example, sesame oil, soybean oil and the like are used, and dissolution-assisting agents such as benzyl benzoate and benzyl alcohol may be used in combination. It may be combined with, for example, buffers (e.g. phosphate buffer, sodium acetate buffer, and the like), analgesics (e.g. benzalkonium chloride, procaine hydrochloride and the like), stabilizers (e.g. human serum albumin, polyethylene glycol and the like), preservatives (e.g. benzyl alcohol, phenol and the like), antioxidants and the like. The preparated solution for injection is usually filled in appropriate ampules.

[0139] Since the preparations obtained as described above are safe and low toxic, they can be administered to, for example, humans and warm-blooded animals (e.g. rats, mice, guinea pigs, rabbits, birds, sheeps, pigs, bovines, cats, dogs, monkeys).

[0140] The dosage of said protein or DNA differs depending on the symptoms and the like. When it is administered orally, in general, for adult patients (60 kg body weight), for example, about 0.1 mg - 100 mg per day, preferably about 1.0 mg - 50 mg per day, more preferably about 1.0 mg - 20 mg per day is administered. When it is administered non-orally, the dosage per dosing differs depending on the target individual, target organ, symptom, administration method and the like. For example, in case of injection, to adult patients (60 kg body weight), for example, it is desirable to intravenously inject about 0.01 - 30 mg per day, preferably about 0.1 - 20 mg per day, more preferably about 0.1 - 10 mg per day. The protein can be administered to other animals, too, in a dose corresponding to the dosage converted for use for 60 kg.

(2) Gene diagnostic agent

[0141] Since, using the DNA of this invention as a probe, abnormalities (gene aberration) in the DNA encoding the protein or its partial peptides of this invention can be detected in humans and warm-blooded animals (e.g., rats, mice, guinea pigs, rabbits, sheep, pigs, bovines, horses, cats, dogs, monkeys and the like), the DNA is useful as gene diagnostic agents for diseases in which the protein of this invention is involved.

[0142] For example, when damage or deficiency of a DNA or mRNA encoding the protein of this invention or a partial peptide thereof, or decrease in the expression of the protein is detected, for example, the disease may be diagnosed as hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure and the like.

[0143] On the other hand, when increase of DNA or mRNA encoding the protein of this invention or a partial peptide thereof, or increase in expression of the protein is detected, for example, the disease may be diagnosed as oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure and the like.

[0144] The gene diagnosis using the DNA of this invention described above can be performed by currently known methods, such as, northern hybridization and PCR-SSCP (Genomics Vol. 5, 874-879 (1989); Proceedings of the National Academy of Sciences of the United States of America Vol. 86, 2766-2770 (1989)) or the like.

(3) Quantification of the protein, its partial peptides, and their salts of this invention

[0145] Since the antibodies of this invention specifically recognize the protein of this invention, the antibodies can be used to quantify the protein of this invention in test solutions, especially for a quantification of said protein by the sandwich immunoassay.

[0146] This invention provides, for example, the following quantification methods:

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tified. When the amount of antigen in the test solution is small and only a small amount of precipitate is obtained, for example, laser nephrometry using scattering of laser is appropriately used.

[0158] For applying these immunological methods to the quantification methods for this invention, no specific conditions, procedures or the like are necessary. Systems for measuring the protein of this invention are constructed by adding the usual technical consideration in the art to the conventional conditions and procedures. The details of these general technical means can be referred to reviews and texts.

[0159] For example, Irie, H. ed. 'Radioimmunoassay' (Kodansha, 1974), Irie, H. ed. 'Sequel to the Radioimmunoassay' (Kodansha, 1979), Ishikawa, E. et al. ed. 'Immunoenzyme assay' (Igakushoin, 1978), Ishikawa, E. et al. ed. 'Immunoenzyme assay' (2nd ed.) (Igakushoin, 1982), Ishikawa, E. et al. ed. 'Immunoenzyme assay' (3rd ed.) (Igakushoin, 1987), Methods in ENZYMOLOGY Vol. 70 (Immunochemical Techniques (Part A)), Vol. 73 (Immunochemical Techniques (Part B)), Vol. 74 (Immunochemical Techniques (Part C)), Vol. 84 (Immunochemical Techniques (Part D: Selected Immunoassays)), Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods)), Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies)) (Academic Press Publishing) and the like can be referred.

[0160] By using the antibodies of this invention as mentioned above, the protein and the like of this invention can be quantified with high sensitivity.

[0161] Moreover, by quantitative determination of the concentration of the protein of this invention using the antibody of this invention, various diseases involving the protein of this invention can be diagnosed.

[0162] For example, when the concentration of the protein of this invention shows a decrease, the disease may be diagnosed as hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure and the like.

[0163] In contrast, when the concentration of the protein of this invention shows an increase, the disease may be diagnosed as oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure and the like.

[0164] In addition, among the antibodies of this invention, the antibody capable of neutralizing the activity of the protein of this invention can be used as a pharmaceutical agent such as an agent for the prophylaxis or treatment of a disease such as oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure and the like, and the like.

[0165] Furthermore, the antibody of this invention can be used to detect the protein of this invention and the like present in a test sample such as body fluid, tissue and the like. In addition, it can be used for the preparation of an antibody column to be used for purification of the protein of this invention and the like, and detection of the protein of this invention and the like in each fraction during the purification.

(4) Screening of candidate compound for pharmaceutical agent

(A) Screening method for receptor agonist or antagonist

[0166] The protein of this invention can specifically bind to a phosphatonin receptor (hereinafter to be briefly referred to as receptor) present on a renal tubular cell. Therefore, by constructing a ligand-receptor binding assay system using the protein of this invention and said receptor, screening of a candidate compound for pharmaceutical agent, which has a similar action with the protein of this invention and screening of a candidate compound for pharmaceutical agent, which inhibits the action of the protein of this invention, can be performed. Thus, this invention provides a screening method for a receptor agonist or antagonist using the protein of this invention.

[0167] More specifically, this invention provides

(1) a screening method for a receptor agonist or antagonist, which comprises comparing between (i) a case where the protein of this invention is brought into contact with the receptor or a partial peptide thereof, and (ii) a case where the protein of this invention and the like and a test compound are brought into contact with the receptor or a partial peptide thereof, and

(2) a screening method for a receptor agonist or antagonist, which comprises comparing between (i) a case where the protein of this invention and the like is brought into contact with a cell containing a receptor or cell membrane fraction thereof, and (ii) a case where the protein of this invention and a test compound are brought into contact with a cell containing a receptor or cell membrane fraction thereof.

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[^{35}S] and the like, and the like can be used.

[0177] As a cell containing the above-mentioned receptor, which is used for the screening method of this invention, those similar to the ones recited as the aforementioned host cell to be used for the expression of protein of this invention can be used, with preference given to CHO cells and the like. The cell containing a receptor can be produced using a DNA encoding the receptor according to publicly known method(s), such as the aforementioned method for expressing the protein of this invention, and the like. As a cell containing the above-mentioned receptor, a cell line such as CL8 cell (BONE, 18, 159-169, 1996), OK cell (AMERICAN JOURNAL OF PHYSIOLOGY, 253, E221-E227, 1987) and the like can be also used.

[0178] When a cell containing a receptor is used for the screening method of this invention, said cell can be immobilized with glutaraldehyde, formalin and the like. The immobilization can be done by publicly known method(s).

[0179] Cell membrane fraction is a fraction abundant in cell membranes obtained by publicly known methods after disruption of the cells. The cell disruption methods include crush of the cells with a Potter-Elvehjem homogenizer, crush using a Waring blender or polytron (Kinematica Co.), disruption by ultrasonication, and disruption by passing the cells through a narrow nozzle with compressing the cells using a French Press. For the cell membrane fractionation, fractionation based on centrifugal force such as a centrifugation for fractionation and a density gradient centrifugation are mainly used. For example, disrupted cell suspension is centrifuged at a low speed (500 - 3,000 rpm) for a short time (usually about 1 - 10 minutes), then the supernatant is centrifuged at a high speed (15,000 - 30,000 rpm) for usually 30 minutes - 2 hours, and the obtained precipitate is used as the membrane fraction. The membrane fraction rich in membrane components such as the expressed receptor and the protein of this invention, and phospholipids and membrane proteins derived from the cells.

[0180] For the amount of the receptor expressed on the cells containing the receptor or the membrane fraction thereof, 10^3 - 10^8 molecules per cell is preferred, and 10^5 - 10^7 molecules per cell is appropriate. As the expression level increases, the ligand-binding activity (specific activity) of the membrane fraction increases, which allows not only construction of a highly sensitive screening system but also measurement of a large number of samples using the same lot.

[0181] For the test compounds, for example, proteins, non-proteinous compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, and animal tissue extracts, and the like are used, and these compounds may be novel or publicly known compounds.

[0182] In the screening method of this invention, the reaction between the protein of this invention and a receptor can be performed generally in about 37°C for several hours.

[0183] Specifically, in order to perform the screening method of the above-mentioned (1a) or (2a), at first, a cell containing a receptor or a cell membrane fraction thereof, a receptor or a partial peptide thereof of this invention is suspended in a buffer suitable for screening to give a receptor preparation. For the buffer, any buffer that does not inhibit the binding of the protein of this invention to the receptor, such as phosphate buffer and Tris-hydrochloride buffer (pH ca. 4 - 10, preferably pH ca. 6 - 8) can be used. To reduce non-specific binding, a surfactant such as CHAPS, Tween-80™ (Kao-Atras Co.), digitonin, and deoxycholate may be added to the buffer. Furthermore, in order to suppress degradation of the receptor and its ligands by proteases, protease inhibitors such as PMSF, leupeptin, bacitracin, aprotinin, E-64 (Institute for Protein Research), and pepstatin and the like may be added. On the other hand, when the cell is an adhesive cell, the protein of this invention and the like can be bound to the receptor by using a cell adhered on a culture vessel, that is the condition the cell is alive, or by using a cell fixed on the culture vessel with glutaraldehyde or paraformaldehyde.

[0184] In these cases, medium, Hanks' solution and the like are used as said buffer. A specified amount (e.g., about 10,000 - 1,000,000 cpm in the case of 2000Ci/mmol) of labeled protein of this invention and the like (e.g., [^{125}I] labeled protein of this invention) is added to 0.01 - 10 ml of the receptor solution, with simultaneous addition of 10^{-4} M - 10^{-10} M test compound. To examine the non-specific binding (NSB), reaction tubes containing a highly excess amount of the non-labeled protein of this invention are also prepared. The reaction is performed at 0°C - 50°C, preferably 4°C - 37°C, for 20 minutes - 24 hours, preferably for 30 minutes - 3 hours. After the reaction, the reaction mixture is filtrated through a glass fiber filter and the filter was washed with an appropriate volume of the same buffer. The radioactivity (e.g., amount of [^{125}I]) remaining on the glass fiber filter is measured by using a liquid scintillation counter or γ-counter. For filtration, Manifold and cell harvester can be used, wherein use of a cell harvester is desirable to achieve higher efficiency. Regarding the count obtained by subtracting the amount of non-specific binding (NSB) from the count obtained in the absence of competitive substance (B_0) as 100%, when the amount of specific binding (B-NSB) is, for example, 50% or less of the count (B_0-NSB), the test compound can be selected as a candidate compound for agonist or antagonist.

[0185] Furthermore, to perform the screening method of the above-mentioned (2b), the cell stimulating activity via a receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like), Na^+-Pi transport activity, 25-hy-

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[Measurement method]

[0197]

(i) CHO cells containing a recombinant receptor are cultured in 12-well culture plates and washed twice with 1 ml of the measurement buffer, and 490 μl of the measurement buffer is added to each well.
(ii) After adding 5 μl of 10^{-3} - 10^{-10} M test compound solution, 5 μl of labeled protein of this invention (5 nM) is added, and the cells are incubated at room temperature for one hour. In order to estimate the non-specific binding, 5 μl of the protein of this invention (10^{-4} M) is added in place of the test compound.
(iii) The reaction solution is removed, and the wells are washed three times with 1 ml of the washing buffer. The labeled protein of this invention bound to the cells is dissolved with 0.5 ml of 0.2N NaOH-1% SDS, and mixed with 4 ml of liquid scintillator A (Wako Pure Chemical Industries, Ltd.)
(iv) The radioactivity is measured using a liquid scintillation counter (Beckman Co.), and the percent maximum binding (PMB) is calculated by the equation [Equation 1] below. When labeled with [^{125}I], it can be directly measured on a gamma counter, without mixing with a liquid scintillator.

[Equation 1]

$$PMB = [(B - NSB)/(B_0 - NSB)] \times 100$$

PMB : Percent maximum binding
B : Binding amount obtained in the presence of test compound
NSB : Non-specific binding
B_0 : Maximum binding

[0198] As mentioned above, the protein of this invention is useful as a reagent for screening a receptor agonist or antagonist.

[0199] The compound or a salt thereof obtained using the screening method or screening kit of this invention is the compound which inhibits binding the protein of this invention to its receptor, specifically, the compound or a salt thereof (so-called, receptor agonist) having an action such as the cell stimulating activity via said receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like), Na^+-Pi transport inhibitory activity, 25-hydroxy vitamin D_3-1_α-hydroxylase inhibitory activity, 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity and the like, or the compound , or a salt thereof (so-called, receptor antagonist) without an action such as the cell stimulating activity via a receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like), Na^+-Pi transport inhibitory activity, 25-hydroxy vitamin D_3-1_α-hydroxylase inhibitory activity, 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity and the like.

[0200] Because a receptor agonist has the entire or partial physiological activity that the protein of this invention has, it is useful as a safe and low toxic pharmaceutical agent depending on said physiological activity. For example, it is useful for pharmaceutical agent such as an agent for the prophylaxis or treatment and the like of a disease (e.g., hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure and the like).

[0201] In contrast, a receptor antagonist can suppress the entire or partial physiological activity that the protein of this invention has. Therefore, it is useful as a safe and low toxic pharmaceutical agent that suppresses said physiological activity. For example, it is useful for a pharmaceutical agent such as an agent for the prophylaxis or treatment and the like of a disease (oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure and the like).

(B) Screening method or a screening kit for an inhibitor of a proteinase that degrades the protein of this invention

[0202] The protein of this invention or a salt thereof is considered to be inactivated upon cleavage by a proteinase

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recited above can be used.

[0215] For the test compound, for example, protein, non-proteinous compound, synthetic compound, fermentation product, cell extract, plant extract, animal tissue extract and the like are used, and these compounds may be novel or publicly known compounds.

[0216] In the screening method of this invention, incubation of proteinase and the protein of this invention can be done generally for several hours at about 37°C. The reaction of this reaction mixture with a cell containing a receptor can be performed generally for several hours at about 37°C.

[0217] The cell stimulating activity via a receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like), Na^+-Pi transport activity, 25-hydroxy vitamin D_3-1_α-hydroxylase activity, 25-hydroxy vitamin D_3-24-hydroxylase activity and the like can be measured in the same manner as in the aforementioned.

[0218] The screening kit of this invention contains the protein of this invention and a proteinase that degrades the protein of this invention, and preferably further contains a cell containing a receptor.

[0219] Examples of the screening kit of this invention include the following.

[Reagent for screening]

(1) Buffers for measurement and washing

[0220] Hanks' balanced salt solution (Gibco Co.) supplemented with 0.05% bovine serum albumin (Sigma Co.). The solution is sterilized by filtration through a 0.45 μm (pore size) filter, and stored at 4°C or may be prepared at use.

(2) Receptor preparation

[0221] CHO cells containing the receptor to the protein of this invention and the like which were seeded in 12-well plates at a density of 5×10^5 cells/well and cultured at 37°C under 5% CO_2 and 95% air for two days.

(3) Preparation of the protein of this invention

[0222] The protein of this invention, a partial peptide thereof or a salt thereof.

(4) Preparation of proteinase that degrades the protein of this invention

[0223] A proteinase that degrades the protein of this invention.

[Measurement method]

[0224]

(1) A proteinase that degrades the protein of this invention and the protein of this invention are incubated at about 37°C for several hours.
(2) A proteinase that degrades the protein of this invention, a test compound and the protein of this invention are incubated at about 37°C for several hours.
(3) The reaction mixtures obtained in the above-mentioned (1) and (2) are respectively cultured together with a cell containing a receptor to the protein of this invention at about 37°C for several hours.
(4) Then, the cell stimulating activity via said receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like), Na^+-Pi transport activity, 25-hydroxy vitamin D_3-1_α-hydroxylase activity, 25-hydroxy vitamin D_3-24-hydroxylase activity and the like are measured according to the aforementioned method.

[0225] As mentioned above, the protein of this invention is useful as a reagent for screening a compound or a salt thereof having an inhibitory activity on a proteinase that degrades the protein of this invention.

[0226] The compound or a salt thereof obtained using the screening method or screening kit of this invention is the compound that inhibits a proteinase that degrades the protein of this invention, and suppresses inactivation of the protein of this invention by said protease. Therefore, said compound can promote activities such as a cell stimulating activity via said receptor (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} con-

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can be used.

[0238] As a cell containing the above-mentioned receptor, which is used for the screening method of this invention, those similar to the ones recited above as the aforementioned host cell to be used for the expression of protein of this invention can be used. Of those recited, CHO cells and the like are preferable. The cell containing a receptor can be produced using a DNA encoding the receptor according to publicly known method(s), such as the aforementioned method for expressing the protein of this invention, and the like. As a cell containing the above-mentioned receptor, a cell line such as CL8 cell (BONE, 18, 159-169, 1996), OK cell (AMERICAN JOURNAL OF PHYSIOLOGY, 253, E221-E227, 1987) and the like can be also used.

[0239] When a cell containing a receptor is used for the screening method of this invention, said cell can be immobilized with glutaraldehyde, formalin and the like. The immobilization can be done by publicly known method(s).

[0240] As a cell membrane fraction containing the above-mentioned receptor, those similar to the ones recited above can be used.

[0241] For the test compounds, for example, proteins, non-proteinous compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, animal tissue extracts and the like are used, and these compounds may be novel or publicly known compounds.

[0242] In the screening method of this invention, the reaction between the protein of this invention and a receptor can be performed generally in about 37°C for several hours.

[0243] In the screening method of the above-mentioned (1a), the cell stimulating activity (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like) induced by the protein of this invention via a receptor, can be measure in the same manner as above.

[0244] In the screening method of the above-mentioned (1a), when the cell stimulating activity (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change in cell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like) induced by the protein of this invention via said receptor, is promoted by the addition of a test compound, said test compound can be selected as a compound or a salt thereof that promotes intracellular signal transduction after the binding of the protein of this invention to the receptor. On the other hand, when the cell stimulating activity (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate, change incell membrane potential, phosphorylation of intracellular protein, lowering of pH and the like) induced by the protein of this invention via a receptor is inhibited by the addition of a test compound, said test compound can be selected as a compound or a salt thereof that promotes intracellular signal transduction after the binding of the protein of this invention to the receptor.

[0245] The screening kit of this invention contains the protein of this invention, and preferably further contains a cell containing a receptor.

[0246] Examples of the screening kit of this invention include the following.

[Reagent for screening]

(1) Buffers for measurement and washing

[0247] Hanks' balanced salt solution (Gibco Co.) supplemented with 0.05% bovine serum albumin (Sigma Co.). The solution is sterilized by filtration through a 0.45 μm (pore size) filter, and stored at 4°C or may be prepared at use.

(2) Receptor preparation

[0248] CHO cells containing the receptor to the protein of this invention which are seeded in 12-well plates at a density of 5 x 10^5 cells/well and cultured at 37°C under 5% CO_2 and 95% air for two days.

(3) Preparation of the protein of this invention

[0249] The protein of this invention, a partial peptide thereof or a salt thereof.

[Measurement method]

[0250] The cell stimulating activity (e.g., release of arachidonic acid, release of acetylcholine, change in intracellular Ca^{2+} concentration, intracellular production of cAMP, intracellular production of cGMP, production of inositol phosphate,

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after to be briefly referred to as a transgenic animal of this invention) can be created by transferring the objective exogenous DNA of this invention into an unfertilized egg, a fertilized egg, sperm, a germinal cell including a progenitor cell thereof and the like, preferably in the stage of embryogenesis in a non-human mammal (more preferably in the stage of single cell or fertilized ovum, and generally before 8 cell stage) according to calcium phosphate method, electric pulse method, lipofection method, aggregation method, microinjection method, particle gun method, DEAE-dextran method or the like. By transferring the objective exogenous DNA of this invention to somatic cell, organs of organisms, tissue cell and the like by said DNA transfer method, they can be utilized for cell culture, tissue culture and the like. Moreover, a transgenic animal of this invention can also be created by fusing these cells with the aforementioned germinal cells according to publicly known cell fusion method.

[0262] As the non-human mammal, for example, bovine, pig, sheep, goat, rabbit, dog, cat, guinea pig, hamster, mouse, rat and the like are used. Among them, from the aspects of preparation of pathological animal models, rodent whose developing period and life cycle is comparatively short and that are easily bred, particularly mice (e.g., as pure strain, C57BL/6 strain, DBA2 strain and the like, and as cross strain, B6C3F1 strain, BDF1 strain, B6D2F1 strain, BALB/c strain, ICR strain and the like) or rats (e.g., Wistar, SD and the like) and the like are preferable.

[0263] As the "mammal" for the expression of the recombinant vector, human and the like can be mentioned besides the above-mentioned non-human mammals.

[0264] The exogenous DNA of this invention is not a DNA of this invention that non-human mammals have, but DNA of the present invention, which is once isolated and extracted from the mammal.

[0265] The mutant DNA of this invention is one that shows a variation in the base sequence of DNA of this invention (e.g., mutant and the like), which is specifically a DNA wherein addition, deletion of bases and substitution by different base, and the like occurred, and the like, including abnormal DNA.

[0266] As said abnormal DNA, a DNA that expresses abnormal protein of this invention, for example, a DNA that expresses a protein that suppresses normal function of the protein of this invention, and the like are used.

[0267] The exogenous DNA of this invention may be derived from a mammal homogeneous or heterogeneous to the target animal. For transfer of the DNA of this invention to a target animal, it is generally beneficial to use a DNA construct wherein the DNA is ligated to the downstream of a promoter that can express said DNA in an animal cell. For example, when human DNA of this invention is transferred, a DNA construct (e.g., vector and the like) wherein the human DNA of this invention is ligated to the downstream of various promoters that can express DNA derived from various mammals (e.g., rabbit, dog, cat, guinea pig, hamster, rat, mouse and the like) possessing the DNA of this invention having high homology therewith, is microinjected into a fertilized egg of the target mammal, for example, mouse fertilized egg, whereby a transgenic mammal that is capable of high expression of the DNA of this invention can be created.

[0268] As the expression vector of the protein of this invention, plasmids derived from *Escherichia coli,* plasmids derived from *Bacillus subtilis*, plasmids derived from yeast, bacteriophages such as λ phage, retroviruses such as Moloney leukemia virus, animal viruses such as vaccinia virus and baculoviruses, and the like are preferably used. Among them, plasmids derived from *Escherichia coli,* plasmids derived from *Bacillus subtilis* and plasmids derived from yeast and the like are preferably used.

[0269] As the promoter to regulate the expression of the above-mentioned DNA, for example, promoters of DNA derived from virus (e.g., simian virus, cytomegalovirus, Moloney leukemia virus, JC virus, breast cancer virus, poliovirus and the like) and as those derived from various mammals (human, rabbit, dog, cat, guinea pig, hamster, rat, mouse and the like) and avians (chicken and the like), promoters of albumin, insulin II, uroplakin II, elastase, erythropoietin, endothelin, muscle creatine kinase, glial fiber acidic protein, glutation S-transferase, platelet-derived growth factor β, keratin K1, K10 and K14, type I and type II collagen, cyclic AMP-dependent protein kinase βI subunit, dystrophin, tartaric acid-resistant alkaline phosphatase, atrial natriuretic factor, endothelial receptor tyrosine kinase (generally abbreviated as Tie2), sodium potassium adenosine triphosphatase (Na, K-ATPase), neurofilament light chain, metallothionein I and IIA, metalloproteinase 1, tissue inhibitor, MHC class I antigen (H-2L), H-ras, renin, dopamine β-hydroxylase, thyroid peroxidase (TPO), polypeptide chain elongation factor 1_α (EF-1_α), β-actin, α and β myosin heavy chain, myosin light chain 1 and 2, myelin basic protein, thyloglobulin, Thy-1, immunoglobulin, H-chain variable region (VNP), serum amyloid P component, myoglobin, troponin C, smooth muscle α actin, preproenkephalin A, vasopressin and the like, are used. Preferably, cytomegalovirus promoter, promoter of human polypeptide chain elongation factor 1_α (EF-1_α), human and chicken β actin promoter and the like, which are capable of systemic high expression of the target DNA, can be used.

[0270] The above-mentioned vector preferably contains a sequence (generally called a terminator) that terminates transcription of the objective messenger RNA in a transgenic mammal. For example, virus-derived, various mammals and avians-derived respective DNA sequences can be used, and preferably, SV40 terminator of simian virus and the like are used.

[0271] In addition, with the aim of affording still higher expression of the objective DNA, splicing signal, enhancer region, partial intron of eucaryotic DNA and the like of each DNA may be connected to 5' upstream of promoter region,

example,

(1) use as cell source for tissue culture,
(2) analysis of relationship with a protein that is specifically expressed or activated due to the protein of this invention, by a direct analysis of DNA or RNA in the tissue of the transgenic animals of this invention or by analysis of protein expressed by the DNA in the tissue,
(3) study of cell function from tissue generally difficult to culture, by culturing cells of tissue having a DNA by general tissue culture technique and using them,
(4) screening of a pharmaceutical agent that enhances the function of cells by the use of the cell described in the above-mentioned (3), and
(5) isolation and purification of mutant protein of this invention and production of its antibody, and the like.

[0284] Furthermore, clinical condition of the diseases relating to the protein of this invention, including functionally inactive adiaphoria to the protein of this invention can be investigated using the transgenic animal of this invention, and detailed pathological findings in each organ of the disease model relating to the protein of this invention can be obtained, thus contributing to the development of a new therapeutic method, and study and therapy of secondary disease due to said disease.

[0285] Establishment of cultured cell is also possible by removing each organ from the transgenic animal of this invention, followed by dicing, liberating DNA-transferred cell by a protease such as trypsin, and culturing thereof. Moreover, characterization of a cell producing the protein of this invention, relationship with hypophosphatemia, or signal transduction mechanism thereof can be examined and look for abnormality therein and the like, thus providing effective research material for the elucidation of the protein of this invention and its action.

[0286] Furthermore, for the development of a therapeutic medicine of diseases relating to the protein of this invention, including functionally inactive adiaphoria to the protein of this invention, by the use of the transgenic animal of this invention, an effective and rapid screening method of a said therapeutic drug of the disease can be provided, using the aforementioned test method, quantification method and the like. In addition, using the transgenic animal of this invention or an exogenous DNA expression vector of this invention, a DNA therapy of diseases relating to the protein of this invention can be studied and developed.

(6) Preparation of knockout animal

[0287] The present invention further provides a non-human mammal embryonic stem cell where the DNA of this invention is inactivated and non-human mammal deficient in expression of DNA of this invention.

[0288] Accordingly, the present invention provides:

(1) a non-human mammal embryonic stem cell where the DNA of this invention is inactivated,
(2) the embryonic stem cell of (1), which is a cell having β-galactosidase gene derived from *Escherichia coli*,
(3) the embryonic stem cell of (1), which is neomycin-resistant,
(4) the embryonic stem cell of (1), wherein the non-human mammal is a rodent,
(5) the embryonic stem cell of (4), wherein the rodent is a mouse,
(6) Non-human mammal deficient in expression of DNA of the present invention,
(7) the animal of (6), wherein a reporter gene can be expressed under regulation of promoter of the protein of this invention,
(8) the animal of (7), wherein the reporter gene is β-galactosidase gene derived from *Escherichia coli*,
(9) the animal of (6), wherein the non-human mammal is a rodent,
(10) the animal of (7), wherein the rodent is a mouse, and
(11) a method for screening a test compound or a salt thereof that promotes a promoter activity of the protein of this invention, which comprises administering the compound to the animal of (7) and detecting expression of the reporter gene.

[0289] The non-human mammal embryonic stem cell where the DNA of this invention is inactivated means an embryonic stem cell (hereinafter to be briefly referred to as ES cell) of a non-human mammal, wherein the DNA does not substantially have the expression capability of the protein of this invention (hereinafter sometimes to be referred to as knockout DNA of the present invention), which is achieved by artificially introducing a mutation to the DNA of this invention possessed by the non-human mammal to suppress expression capability of DNA, or by substantially obliterating the activity of the protein of this invention that the DNA codes for.

[0290] As the non-human mammal, those similar to the aforementioned can be used.

[0291] As a method for artificially introducing a mutation to the DNA of the present invention, for example, a part or

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by differentiating the ES cell of the present invention, is useful for cell biological investigation of the protein of this invention *in vitro*.

[0301] Non-human mammal deficient in expression of the DNA of this invention can be distinguished from normal animals by measuring and indirectly comparing the expression level of the mRNA of said animal by a publicly known method.

[0302] As the non-human mammal, those similar to the aforementioned can be used.

[0303] The non-human mammal which is deficient in the expression of DNA of the present invention can be produced as follows. For example, a targeting vector prepared as mentioned above is introduced into a mouse embryonic stem cell or mouse ovum, and as a result of the introduction, a DNA sequence in the targeting vector in which the DNA of this invention is inactivated, is replaced with the DNA of this invention on the chromosome of the mouse embryonic stem cell or mouse ovum, by homologous recombination, whereby the DNA of this invention can be knocked out.

[0304] The cell wherein the DNA of this invention is knocked out can be judged by southern hybridization analysis using a DNA sequence on the DNA of this invention or in the vicinity thereof as a probe, or by PCR using, as primers, the DNA sequence on a targeting vector and a DNA sequence in the vicinity that is other than the DNA of the present invention and was used as the targeting vector. When a non-human mammal embryonic stem cell is used, a cell line wherein the DNA of this invention is inactivated by gene homologous recombination is cloned, and the cells are injected at a suitable stage, for example, into 8 cell embryo or blastocyst of non-human mammal, and the chimeric embryo prepared is transplanted into the uterus of the pseudopregnant non-human mammal. The created animal is a chimeric animal consisting of cells having a normal locus of the DNA of the present invention and cells having locus of artificially mutated DNA of the present invention.

[0305] When part of the germ cells of the chimeric animal has the locus of mutant DNA of the present invention, such chimeric individual and a normal individual are mated to give individual group, from which an individual whose entire tissues consist of cells having the locus of DNA of the present invention in which artificial mutation was added, can be obtained by, for example, judgment of coatcolor and the like. The thus-obtained individual is generally a heterozygote which is deficient in the expression of the protein of this invention. The heterozygotes, which is deficient in the expression of the protein of this invention are mated each other and the homozygote which is deficient in the expression of the protein of this invention, can be obtained from their offspring.

[0306] When an ovum is used, for example, a transgenic non-human mammal incorporating a targeting vector in chromosome can be obtained by injecting a DNA solution into an ovum nucleus by a microinjection method, and selecting one that has a mutation in the locus of DNA of this invention by gene homologous recombination, as compared to such transgenic non-human mammal.

[0307] An individual in which the DNA of this invention is knocked-out can be bred over generations in ordinary breeding environment, upon confirmation of knocked-out of said DNA in the individual animal obtained by mating.

[0308] Moreover, establishment and maintenance of germ line can be performed by following the conventional methods. That is, a homozygote animal having said inactivated DNA in both the homologous chromosomes can be obtained by mating male and female animal retaining said inactivated DNA. The homozygote animal thus obtained can be reproduced efficiently by breeding in the state where normal individual is 1 and homozygote are plural relative to a mother animal. By mating male and female heterozygote animals, homozygote and heterozygote animals having said inactivated DNA can be bred over generations.

[0309] Non-human mammal embryonic stem cell wherein the DNA of this invention is inactivated is highly useful for creating the non-human mammal deficient in expression of DNA of the present invention. In addition, a mouse deficient in expression of the protein of this invention lacks various biological activities that can be induced by the protein of this invention. Since it can be a model of the disease caused by inactivation of the biological activity of the protein of this invention, it is useful for the investigation of the cause of such disease and consideration of the treatment methods.

[0310] Moreover, in an animal that expresses the protein of this invention wherein the structural gene of the protein of this invention is substituted by a reporter gene, the reporter gene is present under the control of the promoter of the protein of this invention, so that the activity of the promoter of the protein of this invention can be detected by tracing the expression of the substance that the reporter gene encodes. For example, when a part of a DNA region encoding the protein of this invention is substituted by β-galactosidase gene (lacZ) derived from *Escherichia coli*, β-galactosidase expresses instead of the protein of this invention in a tissue where the protein of this invention inherently expresses. Accordingly, for example, the expression manner of the protein of this invention in an animal in vivo can be conveniently observed by staining using a reagent that becomes a substrate of β-galactosidase such as 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal). To be specific, a mouse deficient in the protein of this invention or the tissue section is immobilized with glutaraldehyde and the like. After washing with Dulbecco's phosphate buffer saline (PBS) and reacting with a staining solution containing X-gal at room temperature or around 7°C for about 30 min to 1 hr, the tissue sample is washed with a 1 mM EDTA/PBS solution to terminate the β-galactosidase reaction and the color development may be observed. In addition, mRNA encoding lacZ may be detected according to a conventional method.

[0311] Such animal deficient in expression of the protein of this invention is extremely useful for screening a sub-

Z :	benzyloxycarbonyl
Cl-Z :	2-chlorobenzyl oxycarbonyl
Br-Z :	2-bromobenzyl oxycarbonyl
Boc :	t-butoxycarbonyl
DNP :	dinitrophenol
Trt :	trityl
Bum :	t-butoxymethyl
Fmoc :	N-9-fluorenyl methoxycarbonyl
HOBt :	1-hydroxybenztriazole
HOOBt :	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HONB :	1-hydroxy-5-norbornene-2,3-dicarboxyimide
DCC :	N,N'-dicyclorohexylcarbodiimide

[0314] The SEQ IDs in the sequence listing of this specification present the following sequences.

[SEQ ID:1]

[0315] The amino acid sequence of the protein of this invention, which is derived from human.

[SEQ ID:2]

[0316] The base sequence of a DNA encoding the protein of this invention, which is derived from human.

[SEQ ID:3]

[0317] The base sequence of a DNA containing a DNA encoding the protein of this invention derived from human, which is inserted in plasmid pCR-PHOS.

[SEQ ID:4]

[0318] The base sequence of a primer used for cloning a DNA encoding the protein of this invention, which is derived from human.

[SEQ ID:5]

[0319] The base sequence of a primer used for cloning a DNA encoding the protein of this invention derived from human.

[SEQ ID:6]

[0320] The base sequence of a primer used for cloning a DNA encoding the protein of this invention derived from human.

[SEQ ID:7]

[0321] The base sequence of an Oligo$(dT)_{18}$ linker-primer containing XhoI site used in Example 1.

[SEQ ID:8]

[0322] The base sequence of a primer used for cloning a DNA encoding the protein of this invention derived from human.

[SEQ ID:9]

[0323] The base sequence of a primer used for cloning a DNA encoding the protein of this invention derived from human.

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5'-TCAGGTGGCTCTCCTCTACATCAACTCACA-3'

(SEQ ID:5)

[0333] The PCR reaction was carried out in a system containing TaKaRa Ex Taq (Takara Shuzo Co., Ltd), and using a Thermal Cycler (GeneAmp PCR System, PE Applied Biosystems) under the conditions of 1 cycle at 95°C for 3 min, 30 cycles at 95°C for 45 sec, 58°C for 45 sec and 72°C for 3 min, 1 cycle at 72°C for 5 min, and standing at 4°C.

[0334] The amplified fragment thus obtained was subjected to an electrophoresis using 1% agarose gel, and the PCR products in a main band was extracted and purified, inserted into pCRII-TOPO vector (Invitrogen) using a TOPO TA cloning kit (Invitrogen) and introduced into *Escherichia coli* TOP10 strain.

[0335] Plasmid DNA was extracted from the obtained transformant, subjected to PCR reaction using BigDye Terminator Cycle Sequence Ready Reaction Kit (PE Applied Biosystems), and the base sequence of the cDNA fragment was determined by ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems).

[0336] The plasmid DNA retained by the obtained clone #1 had a sequence (1713 bases) containing essentially the same base sequence as the sequence (1290 bases) encoding Val (1st) to Asp (430th) of phosphatonin described in international publication WO 99/60017 of international patent application by Rowe P.S.N., and encoded phosphatonin protein consisting of 525 amino acids.

[0337] The insert sequence of plasmid DNA retained by clone #1 was different by several bases from the insert sequence of phosphatonin gene sequence described in international publication WO 99/60017 of international patent application by Rowe P.S.N. and that of plasmid DNA retained by simultaneously obtained other clones. To determine the true phosphatonin gene sequence, therefore, PCR was performed using Pyrobest DNA polymerase with a higher fidelity. The λ phage library prepared in the above, which was derived from tumor of OHO patients was used as a template and as a primer, synthetic oligo DNA of SEQ ID:6, which starts from the 5' side of initiation codon ATG of the above-mentioned clone A was used as a forward primer and synthetic oligo DNA of SEQ ID:5 was used as a reverse primer.

5'-CTCAAAGATGCGAGTTTTCTGTGTGGGA-3'

(SEQ ID:6)

[0338] The PCR reaction was carried out using a Thermal Cycler (GeneAmp PCR System, PE Applied Biosystems) under the conditions of 30 cycles at 98°C for 10 sec, 56°C for 45 sec and 72°C for 3 min, and standing at 4°C.

[0339] The amplified fragment thus obtained was subjected to a gel electrophoresis using 1% agarose gel, and the PCR products in a main band was extracted and purified, inserted into pCR-Blunt vector (Invitrogen) using a Zero Blunt PCR Cloning Kit (Invitrogen) and introduced into *Escherichia coli* TOP10 strain.

[0340] Plasmid DNA was extracted from the obtained transformed bacteria, subjected to PCR reaction using BigDye Terminator Cycle Sequence Ready Reaction Kit (PE Applied Biosystems), and the base sequence of cDNA fragment was determined by ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems). As a result, a transformant: *Escherichia coli* TOP10/pCR-PHOS (clone #9) containing plasmid pCR-PHOS retaining a DNA encoding the phosphatonin protein of this invention was obtained.

[0341] The plasmid DNA retained by the obtained TOP10/pCR-PHOS (clone #9) had a sequence (1662 bases) presented by SEQ ID:3 containing the sequence (1575 bases) presented by SEQ ID:2, and encoded phosphatonin protein consisting of 525 amino acids presented by SEQ ID:1 (Figs. 1-4). The molecular weight of the protein portion (including signal peptide) of the protein of this invention was 58.4 kDa as deduced from the amino acid sequence.

[0342] The base sequence presented by SEQ ID:3 had a base sequence essentially identical to a sequence (1290 bases) encoding Val (1st) to Asp (430th) of phosphatonin described in international publication WO 99/60017 of international patent application by Rowe P.S.N. and had a novel sequence (285 bases) encoding 95 amino acid sequence starting with Met in the 5' region thereof. In the sequence common to base sequence presented by SEQ ID:3 and base sequence of phosphatonin described in international publication WO 99/60017 of international patent application by Rowe P.S.N., only the 293rd base was different (Rowe P.S.N. publication : G^{1} → SEQ ID:3: C^{293}), along with which the amino acid sequence presented by SEQ ID:1 was different in one residue (Rowe P.S.N. publication: Val^{1} → SEQ ID:1: Leu^{96}).

[0343] The hydrophobicity of the amino acid sequence presented by SEQ ID:1 was assumed by the Kite Doolittle

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[Table 1] (continued)

Motif	Site (SEQ ID:1)	Amino acid seq.
Casein kinase II phosphorylation	30-33	SCVE
	59-62	SSKE
	73-76	SLSE
	103-106	SNKE
	234-237	SDFE
	272-275	TGPD
	289-292	SEAE
	294-297	THLD
	319-322	TRDE
	323-326	TAKE
	333-336	SLVE
	420-423	TLNE
	518-521	SSSE
	520-523	SESD
	522-525	SDGD
cAMP-dependent protein kinase phosphorylation	501-503	RRFS
Tyrosine kinase phosphorylation	135-142	KLHDQEEY

[0345] The protein of this invention presented by SEQ ID:1 had a sugar chain binding site in Asn^{477} - Thr^{480} and Asn^{478} - Arg^{481}, and a glycosaminoglycan binding site in Ser^{256} - Gly^{259}. The protein of this invention presented by SEQ ID:1 had RGD sequence (Arg^{247} - Asp^{249}) involved in cell adhesion. The RGD sequence is known to be present in collagen, vitronectin, fibrinogen, von Willebrand factor and the like, and expected to contribute to the interaction between the protein of this invention and a cell in as well.

[0346] The protein of this invention presented by SEQ ID:1 has many phosphorylation site for protein kinase C, casein kinase II, cAMP-dependent protein kinase and tyrosine kinase (Table 1), and these sites were considered to play some role in the biological activity of the protein of this invention and a partial peptide thereof. In addition, the protein of this invention presented by SEQ ID:1 had many myristoylation site characteristic of phosphorylated glycoproteins such as collagen, vitronectin, fibronectin, osteopontin, dentin-sialophosphoprotein (DSSP) and the like (Table 1). The C-terminal region of the protein of this invention contains a sequence rich in aspartic acid and serine (Asp^{509} - Ser^{522}; DSSESSDSGSSSES), which showed high 79% homology with a sequence repeatedly seen in DSSP, such as Asp^{686}- Ser^{699}; DSSDSSDSSSSSDS. A similar sequence is also present in osteopontin (Asp^{101} - Asp^{116}; DDSHQS-DESHHHSDESD), suggesting a involvement of the protein of this invention in bone formation.

[0347] While phosphatonin is considered to be cleaved by a PHEX gene product, the amino acid sequence presented by SEQ ID:1 has an amino acid sequence (Ala^{327} - Ser^{333}; ADAVDVS) that matches with the substrate specificity of zinc metalloendopeptidase, so that the site (Val^{330} - Asp^{331}) is expected to be cleaved by a PHEX gene product.

[Example 2] Expression of recombinant Phosphatonin in *Escherichia coli*

[0348] A expression plasmid for a mature phosphatonin without a signal sequence (Met^{1} - Ala^{16}) was constructed and expressed in *Escherichia coli.*

[0349] First, a DNA fragment encoding mature phosphatonin was amplified by PCR (98°C × 45 sec, 56°C × 45 sec, 72°C × 3 min, 30 cycles) using pyrobest DNA polymerase (Takara Shuzo Co., Ltd) using oligo DNA comprising 5' terminal sequence of the mature phosphatonin which was added Nde I site and initiation codon ATG, as a forward primer (5'-CATATGGCACCAACATTTCAACCACAGA-3', SEQ ID:8), oligo DNA having a DNA sequence of 3' side of a stop codon as a reverse primer (5'-CTCTCGTCGACATCAACTCACA-3', SEQ ID:9), and plasmid pCR-PHOS-9 for animal cell expression (Example 1) as a template. The obtained PCR product was purified by an electrophoresis using 1% agarose gel, ligated to a pCR Blant vector (Invitrogen), and transformed to TOP10 competent cell. Plasmid DNA

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at a wavelength of 280 nm, and the obtained data was subjected to waveform treatment with a Chromatocorder 21 (System Instruments Co., Ltd.) and the purity was calculated. As a result, Phosphatonin showed a single peak. From this, the Phosphatonin sample was proved to be homogenious peak and to have a quite high purity (Fig. 8).

[0356] The amino acid composition of the obtained Phosphatonin was determined by an amino acid analyzer (HITACHI L-8500A). The results are shown in [Table 2].

[0357] The measurements of the sample matched with the theoretical value of the amino acid composition of Phosphatonin having Met on the N-terminal.

[Table 2]

Amino acid	Value expected from base sequence of Phosphatonin	Number of residue per mole
Asx	74	70.4
Thr[1)]	23	21.9
Ser[1)]	56	39.9
Glx	70	71.7
Pro	28	29.2
Gly	46	45.0
Ala	22	21.5
Cys[2)]	1	N.D
Val	13	13.2
Met	7	7.4
Ile	25	23.1
Leu	21	21
Tyr	12	12.0
Phe	13	12.1
Lys	50	48.1
His	18	17.8
Trp	1	0.6
Arg	29	25.5

Acid hydrolysis (6N HCl-4% thioglycolic acid, 110°C, average value of 24 and 48 hr of hydrolysis)
1) value extrapolated to 0 hr
2) not detected

Analysis was done by using about 10 μg.

[0358] The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Applied Biosystems model 492). As a result, while the obtained Phosphatonin was a mixture of proteins with and without the N-terminal methionine, the sequence matched with the N-terminal amino acid sequence of Phosphatonin assumed from the base sequence (Fig. 9).

[0359] As shown in Example 1, Phosphatonin has a motif susceptible to modification with phosphorylation. Therefore, phosphorylation with casein kinase II was done. The aforementioned purified Phosphatonin (7.5 mg) was diluted with 20 mM N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES)/NaOH containing 15 mM NaCl, 15 mM $MgCl_2$, and 0.3 mM ATP (pH 7.5) and pre-incubated in a thermobath at 37°C for 5 min. Then casein kinase II (Calbiochem, 47 mU) was added and the mixture was reacted at 37°C for 60 min. After the completion of the reaction, the mixture was ice-cooled and concentrated by ultrafiltration membrane (Vivaspin 20, molecular weight cut off; 10K (k dalton)) (Sartorius). The concentrated solution was applied to Superdex 200 (10 mm I.D. X 30mm L, 13 μm)(Amersham Pharmacia Biotech) equilibrated with PBS, eluted at a flow rate of 0.5 mL/min and the Phosphatonin-containing fraction was pooled.

[0360] With the aim of confirming phosphorylation of Phosphatonin, the aforementioned SDS polyacrylamide gel electrophoresis was performed (multigel 12.5 (Daiichi Pure Chemicals)). The gel after electrophoresis was stained with Coomassie brilliant blue, and as a result, changes in the molecular weight were observed before and after the reaction, suggesting phosphorylation of Phosphatonin (Fig. 10).

[Example 4] Preparation of polyclonal antibody

[0361] The Phosphatonin obtained in Example 3 was emulsified with Freund's complete adjuvant or incomplete adjuvant, and for immunization, the emulsion containing 1 mg of protein was subcutaneously injected to each Kbl:JW

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min, 30 cycles) was performed by using Pyrobest DNA polymerase (Takara Shuzo). The PCR product was subjected to 1% agarose gel electrophoresis and a band having the objective size was extracted from the gel, and purified. After adding adenine to the 3' terminal, the PCR product was ligated to pTARGET™ Vector (Promega) and transformed to JM109 competent cell. Plasmid DNA was obtained from two positive strain selected by colony PCR, and insert check by digestion with Sac I (Takara Shuzo) and DNA sequence analysis gave a plasmid (pT-PHOSF-11) having a correct insert in the forward direction (Fig. 13).

[0368] After cleaving the expression plasmid for Phohsphatonin-FLAG (pT-PHOSF-11) with restriction enzyme AhdI (Biolabs) at one site, it was introduced into CHO-K1 cell by electropolation method (1×10^7 Cells, Plasmid 10 ug/800 ul PBS, 0.4 cm Cuvette, 960 uF-0.25 kV (Bio-Rad)). After culturing overnight in 10 mL of Ham F12-10% FCS medium (Gibco BRL), cloning of the cells by limited dilution with Ham F12-10% FCS medium containing 550 ug/ml of geneticin (Gibco BRL) was performed. The culture supernatant of each clone was screened using the ELISA system described in Example 5 to give a highly stable expression line (CHO-PHOSF-11-34-24).

[Example 7] Purification of recombinant Phosphatonin from CHO

[0369] The recombinant Phosphatonin from CHO cells was purified using an anti-Phosphatonin antibody column. Fifty milligram of anti-Phosphatonin antibody (Example 4) was coupled onto HiTrap-NHS column (5 mL, Amarsham Pharmasia) to give an anti-Phosphatonin antibody column. The culture supernatant (3 L) of a highly stable expression line (CHO-PHOSF-11-34-24) was adsorbed on the anti-Phosphatonin antibody column equilibrated with 50 mM Tris-HCl (pH 8.0), washed with Tris-HCl containing 0.5 M NaCl (pH 8) at a flow rate of 4 mL/min. The Phosphatonin was eluted with 0.1 M glycine containing 0.5 M NaCl (pH 3.0), and neutralized with 1M Tris-HCl (pH 8.0). The eluate was concentrated and substituted with PBS by ultrafiltration membrane (Vivaspin 20, molecular weight cut off; 10K (k dalton))(Sartorius) to give a preparation (2.2 mg). In the same manner as in Example 3, SDS polyacrylamide gel electrophoresis was conducted, which showed bands at about 60 Kd and at a position of high molecular weight, which was considered to be sugar chain-added form (Fig. 14).

[Example 8] Measurement of activity of recombinant Phosphatonin

[0370] The phosphorus reabsorption inhibitory activity of recombinant Phosphatonin from CHO cell, in the kidney was assayed using a normal human proximal tubule epithelial cell (RPTEC, BioWhittaker). RPTEC (2×10^4/well) was cultured in a 24 well plate using REGM (BioWhittaker) at 37°C, 5% CO_2 for 3 days, and the medium was substituted by fresh RBGM (BioWhittaker) containing 0.1% BSA, followed by cultivation overnight at 37°C, 5% CO_2. The recombinant Phosphatonin from CHO diluted with RBGM containing 0.1% BSA (final concentration 1 μg/mL) was added to the well and the plate was incubated at 37°C, 5% CO_2 for 2 hr. The medium was removed and the each well was washed with 1 mL of a washing buffer (10 mM Tris-HEPES containing 137 mM NaCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 5.4 mM KCl (pH 7.4)). An uptaking buffer (10 mM Tris-HEPES containing 137 mM NaCl, 0.1 mM $KH_2{}^{32}PO_4$ (0.5 mCi/mL), 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 5.4 mM KCl (pH 7.4)) was added by 0.25 mL and the plate was incubated at 37°C for 5 min. Then, the uptaking buffer was removed and the each well was washed 3 times with 1 mL of ice-cooled stopping buffer (14 mM Tris-HEPES containing 137 mM NaCl (pH 7.4)). The cells were solubilized with 0.5N NaOH (0.5 mL) (10 mL was preserved for protein concentration measurement), and mixed with 3 mL of liquid scintillation solution A, then the radioactivity was measured by liquid scintillation counter. Protein concentration was determined by using BCA Protein Assay Reagent (Pierce). As shown in Fig. 15, recombinant Phosphatonin from CHO cell inhibited cellular uptake of phosphate in RPTEC.

Industrial Applicability

[0371] The protein of this invention, a partial peptide thereof or a salt thereof has actions such as phosphaturic activity, hypophosphatemia-inducing activity, Na^+-Pi transport inhibitory activity, 25-hydroxy vitamin D_3-1_α-hydroxylase inhibitory activity and 25-hydroxy vitamin D_3-24-hydroxylase-promoting activity in kidney cell and the like. Therefore, the protein of this invention, a partial peptide thereof or a salt thereof, and DNA of this invention are useful as an agent for the prophylaxis or treatment of diseases such as hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure and the like.

[0372] In addition, the DNA of this invention is useful as a gene diagnostic agent of diseases such as oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis, kidney failure, hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy, kidney failure and

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35 40 45

His Leu Gly Lys Arg Ile Asn Gln Gln Leu Ser Ser Lys Glu Asn Ile
50 55 60

Val Gln Glu Arg Lys Lys Asp Leu Ser Leu Ser Glu Ala Ser Glu Asn
65 70 75 80

Lys Gly Ser Ser Lys Ser Gln Asn Tyr Phe Thr Asn Arg Gln Arg Leu
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Asn Lys Glu Tyr Ser Ile Ser Asn Lys Glu Asn Thr His Asn Gly Leu
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Gly Asp Asp Ala Ile Ser Lys Leu His Asp Gln Glu Glu Tyr Gly Ala

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Claims

1. A protein comprising an amino acid sequence identical or substantially identical to an amino acid sequence consisting of amino acid Nos. 17 - 525 of the amino acid sequence presented by SEQ ID:1, or a salt thereof.

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23. A screening kit for a compound or a salt thereof having an inhibitory action on a proteinase that degrades the protein of claim 1 or the partial peptide of claim 7, which comprises the protein of claim 1, the partial peptide of claim 7 or a salt thereof.

24. A compound or a salt thereof having an inhibitory action on a proteinase that degrades the protein of claim 1 or the partial peptide of claim 7, which is obtained by the screening method of claim 22 or by the use of the screening kit of claim 23.

25. The screening method of claim 19, which comprises measuring and comparing the amount of the protein of claim 1, the partial peptide of claim 7 or a salt thereof bound to a receptor or a partial peptide thereof, between (i) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof is brought into contact with the receptor or a partial peptide thereof, and (ii) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof and a test compound are brought into contact with the receptor or a partial peptide thereof.

26. The screening method of claim 19, which comprises measuring and comparing the amount of the protein of claim 1, the partial peptide of claim 7 or a salt thereof bound to a cell containing a receptor or a cell membrane fraction thereof, between (i) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof is brought into contact with the cell containing the receptor or a cell membrane fraction thereof, and (ii) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof and a test compound are brought into contact with the cell containing the receptor or a cell membrane fraction thereof.

27. The screening method of claim 19, which comprises measuring and comparing a cell stimulating activity via a receptor in a cell containing the receptor, a Na^+-Pi transport activity, a 25-hydroxy vitamin D_3-1_α-hydroxylase activity or a 25-hydroxy vitamin D_3-24-hydroxylase activity, between (i) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof is brought into contact with the cell containing the receptor, and (ii) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof and a test compound are brought into contact with the cell containing the receptor.

28. A pharmaceutical agent comprising a receptor agonist obtained by the screening method of any of claim 19, claim 25, claim 26 and claim 27 or by the use of the screening kit of claim 20.

29. The pharmaceutical agent of claim 28, which is an agent for the prophylaxis or treatment of hyperphosphatemia, arteriosclerosis, acute coronary syndrome, heart failure, stroke, chronic glomerulonephritis, diabetic nephropathy or kidney failure.

30. A pharmaceutical agent comprising a receptor antagonist obtained by the screening method of any of claim 19, claim 25, claim 26 and claim 27 or by the use of the screening kit of claim 20.

31. The pharmaceutical agent of claim 30, which is an agent for the prophylaxis or treatment of oncogenic hypophosphatemic osteomalacia (OHO), X-linked hypophosphatemia (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), vitamin D-resistant rachitis, osteomalacia, osteoporosis, renal osteodystrophy, secondary hyperparathyroidism, Paget's disease, renal Fanconi's syndrome, renal tubular acidosis, cystic fibrosis, fibrous cystic ostitis or kidney failure.

32. The screening method of claim 22, which comprises measuring and comparing a cell stimulating activity via a receptor in a cell containing the receptor, Na^+-Pi transport activity, a 25-hydroxy vitamin D_3-1_α-hydroxylase activity or a 25-hydroxy vitamin D_3-24-hydroxylase activity, between (i) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof is brought into contact with the cell containing the receptor in the presence of a proteinase that degrades the protein of claim 1, the partial peptide of claim 7 or a salt thereof, and (ii) a case where the protein of claim 1, the partial peptide of claim 7 or a salt thereof is brought into contact with the cell containing the receptor in the presence of a proteinase that degrades the protein of claim 1, the partial peptide of claim 7 or a salt thereof and a test compound.

33. A pharmaceutical agent comprising a compound or a salt thereof having an inhibitory action on a proteinase that degrades the protein of claim 1 or the partial peptide of claim 7, which is obtained by the screening method of claim 22 or claim 32 or by the use of the screening kit of claim 23.

34. A method for screening a compound or a salt thereof that promotes or inhibits intracellular signal transduction after

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FIG. 1

```
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ATG CGA GTT TTC TGT GTG GGA CTA CTC CTT TTC AGT GTG ACC TGG GCA GCA CCA
Met Arg Val Phe Cys Val Gly Leu Leu Leu Phe Ser Val Thr Trp Ala Ala Pro
         63          72          81          90          99         108
ACA TTT CAA CCA CAG ACT GAG AAA ACT AAG CAA AGC TGT GTG GAA GAG CAG AGG
Thr Phe Gln Pro Gln Thr Glu Lys Thr Lys Gln Ser Cys Val Glu Glu Gln Arg
        117         126         135         144         153         162
CAG GAA GAA AAA AAC AAA GAC AAT ATT GGT TTT CAC CAT TTG GGC AAG AGA ATA
Gln Glu Glu Lys Asn Lys Asp Asn Ile Gly Phe His His Leu Gly Lys Arg Ile
        171         180         189         198         207         216
AAT CAA GAG CTA TCA TCT AAA GAA AAT ATT GTC CAG GAA AGA AAG AAA GAT TTG
Asn Gln Glu Leu Ser Ser Lys Glu Asn Ile Val Gln Glu Arg Lys Lys Asp Leu
        225         234         243         252         261         270
TCC CTT TCT GAA GCC AGT GAG AAT AAG GGA AGT AGT AAA TCT CAA AAT TAT TTC
Ser Leu Ser Glu Ala Ser Glu Asn Lys Gly Ser Ser Lys Ser Gln Asn Tyr Phe
        279         288         297         306         315         324
ACA AAT AGA CAG AGA CTG AAT AAA GAA TAT AGT ATC AGT AAG AAA GAG AAT ACT
Thr Asn Arg Gln Arg Leu Asn Lys Glu Tyr Ser Ile Ser Asn Lys Glu Asn Thr
        333         342         351         360         369         378
CAC AAT GGC CTG AGG ATG TCA ATT TAT CCT AAG TCA ACT GGG AAT AAA GGG TTT
His Asn Gly Leu Arg Met Ser Ile Tyr Pro Lys Ser Thr Gly Asn Lys Gly Phe
        387         396         405         414         423         432
GAG GAT GGA GAT GAT GCT ATC AGC AAA CTA CAT GAC CAA GAA GAA TAT GGC GCA
Glu Asp Gly Asp Asp Ala Ile Ser Lys Leu His Asp Gln Glu Glu Tyr Gly Ala
        441         450         459         468         477         486
GCT CTC ATC AGA AAT AAC ATG CAA CAT ATA ATG GGG CCA GTG ACT GCG ATT AAA
Ala Leu Ile Arg Asn Asn Met Gln His Ile Met Gly Pro Val Thr Ala Ile Lys
```


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FIG. 3

```
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Pro Glu Arg Glu Glu Asn Gly Gly Asn Thr Ile Gly Thr Arg Asp Glu Thr Ala
        981         990         999        1008        1017        1026
AAA GAG GCA GAT GCT GTT GAT GTC AGC CTT GTA GAG GGC AGC AAC GAT ATC ATG
Lys Glu Ala Asp Ala Val Asp Val Ser Leu Val Glu Gly Ser Asn Asp Ile Met
       1035        1044        1053        1062        1071        1080
GGT AGT ACC AAT TTT AAG GAG CTC CCT GGA AGA GAA GGA AAC AGA GTG GAT GCT
Gly Ser Thr Asn Phe Lys Glu Leu Pro Gly Arg Glu Gly Asn Arg Val Asp Ala
       1089        1098        1107        1116        1125        1134
GGC AGC CAA AAT GCT CAC CAA GGG AAG GTT GAG TTT CAT TAC CCT CCT GCA CCC
Gly Ser Gln Asn Ala His Gln Gly Lys Val Glu Phe His Tyr Pro Pro Ala Pro
       1143        1152        1161        1170        1179        1188
TCA AAA GAG AAA AGA AAA GAA GGC AGT AGT GAT GCA GCT GAA AGT ACC AAC TAT
Ser Lys Glu Lys Arg Lys Glu Gly Ser Ser Asp Ala Ala Glu Ser Thr Asn Tyr
       1197        1206        1215        1224        1233        1242
AAT GAA ATT CCT AAA AAT GGC AAA GGC AGT ACC AGA AAG GGT GTA GAT CAT TCT
Asn Glu Ile Pro Lys Asn Gly Lys Gly Ser Thr Arg Lys Gly Val Asp His Ser
       1251        1260        1269        1278        1287        1296
AAT AGG AAC CAA GCA ACC TTA AAT GAA AAA CAA AGG TTT CCT AGT AAG GGC AAA
Asn Arg Asn Gln Ala Thr Leu Asn Glu Lys Gln Arg Phe Pro Ser Lys Gly Lys
       1305        1314        1323        1332        1341        1350
AGT CAG GGC CTG CCC ATT CCT TCT CGT GGT CTT GAT AAT GAA ATC AAA AAC GAA
Ser Gln Gly Leu Pro Ile Pro Ser Arg Gly Leu Asp Asn Glu Ile Lys Asn Glu
       1359        1368        1377        1386        1395        1404
ATG GAT TCC TTT AAT GGC CCC AGT CAT GAG AAT ATA ATA ACA CAT GGC AGA AAA
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FIG. 5

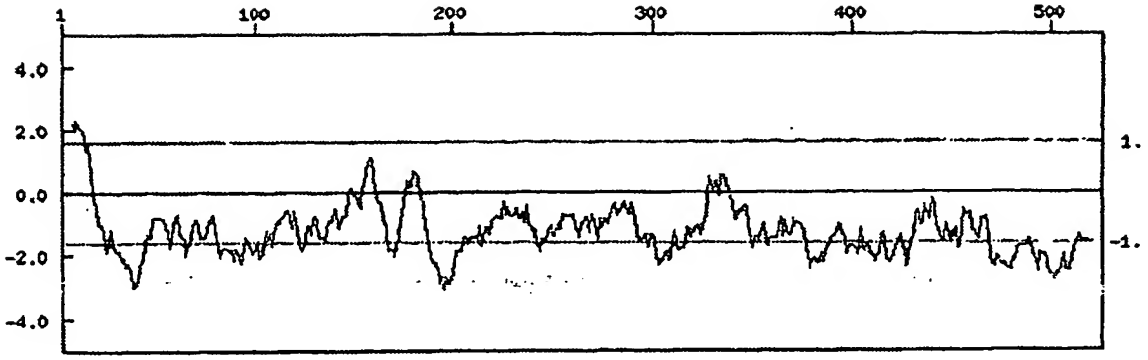

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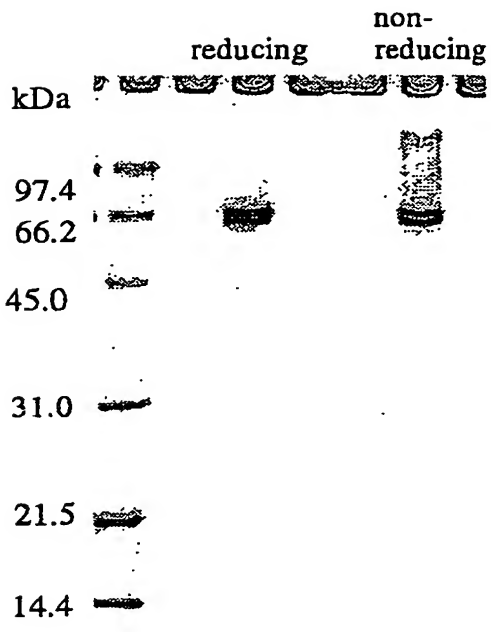
FIG. 7



SDS PAGE
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2min
amount applied
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FIG. 9

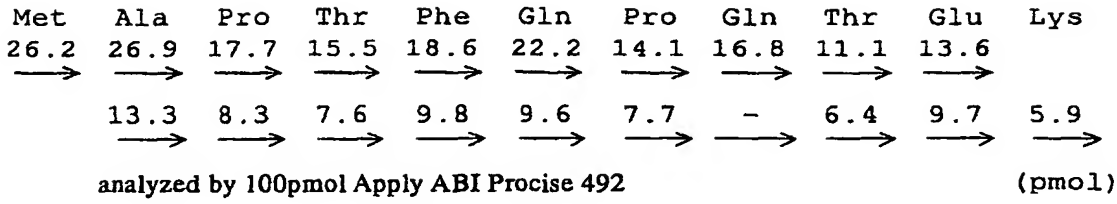

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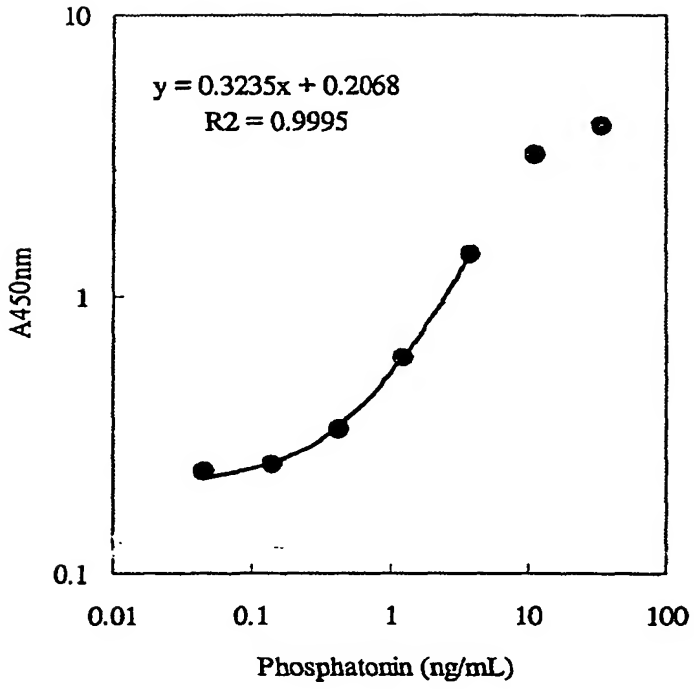
FIG. 11



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detection range: 0.1-3ng/mL (1.8-53fmol/mL)

BNSDOCID: <EP 1293568A1 ! >

FIG. 13

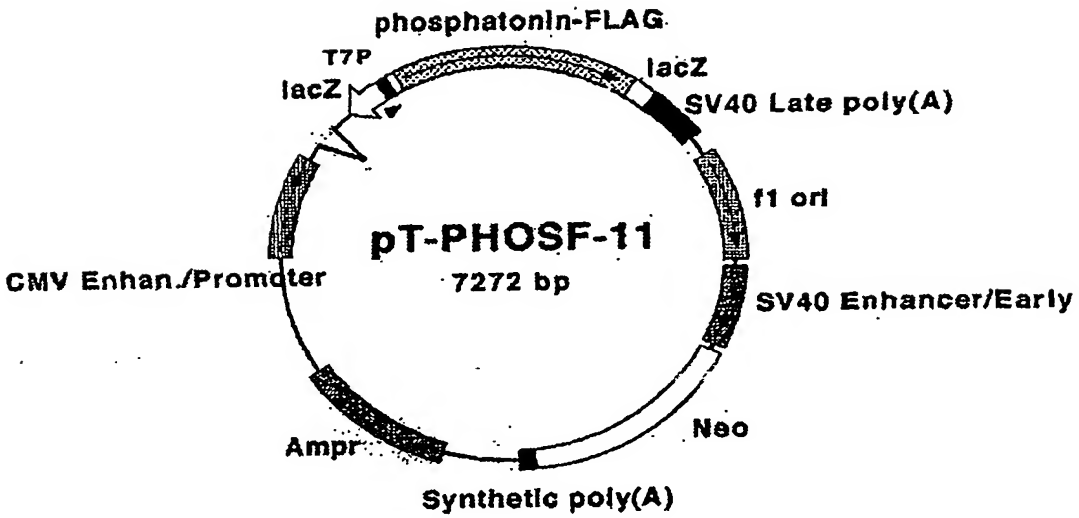

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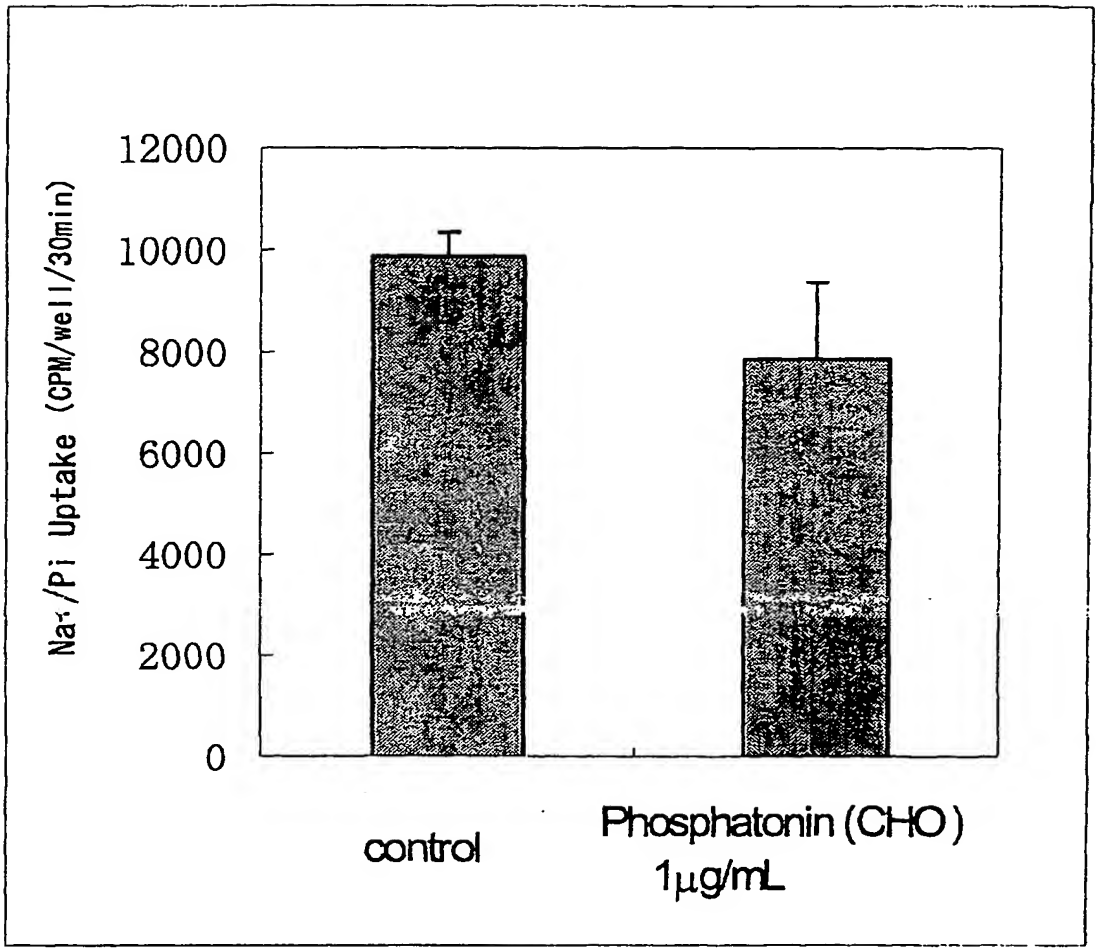
FIG. 15



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/05263

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18
because they relate to subject matter not required to be searched by this Authority, namely:

The invention as set forth in claim 18 pertains diagnostic methods to be practiced on the human body.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

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